

Lack of in vitro resistance of *Candida albicans* to ketoconazole, itraconazole and clotrimazole in women treated for recurrent vaginal candidiasis

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Abstract

Objective—To determine whether in vitro resistance of *Candida albicans* to the imidazoles (ketoconazole, clotrimazole and itraconazole) is associated with recurrence of candida vaginitis.

Design—*Candida* isolates were collected before, during and after treatment from women with recurrent vaginal candidiasis (≥ 4 episodes/year), randomised into two prospective studies: (1) 56 women treated with ketoconazole 400 mg/daily for 7 days; (2) 44 women randomised to receive itraconazole 200 mg orally, or clotrimazole 200 mg intravaginally, twice weekly for six months.

Setting—Women's Candida Clinic at St. Michael's Hospital, a University of Toronto teaching Hospital, Toronto, Ontario, Canada.

Main outcome, measures—Isolates of yeasts recovered pre and post treatment were tested for significant changes in 50% inhibitory concentration (IC_{50}). Resistance was defined as a greater than four-fold increase in baseline IC_{50} of post treatment isolates compared with pre-treatment isolates.

Results—Over 250 strains of *C. albicans* were tested and none showed development of resistance to any of the agents.

Conclusion—Recurrence of vaginal candidiasis is not related to the development of drug resistance.

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Introduction

The pathogenesis of recurrent candida vulvovaginitis in patients with no obvious predisposing factors is poorly understood,¹ and the reasons for the refractoriness of the condition after discontinuation of therapy^{2,3} is unknown. Whether the recurrences of infection represent relapse or reinfection is also undetermined.³ True vaginal relapse due to incomplete eradication of the initial vaginal infection may be responsible for a large percentage of recurrent episodes;⁴ however, the reason for the particular vulnerability of these women to recurrent symptomatic infection is unclear. The possibility of persistence of infection due to development of drug resistant strains exists, but there are little data on the antifungal susceptibility of *Candida albicans* recovered from women with this condition. Recent reports of the development of in vitro resistance to ketoconazole and fluconazole in patients with the acquired

immunodeficiency syndrome (AIDS) and oropharyngeal candidiasis,^{5,6} underscores the importance of this possibility in women with chronic recurrent vaginal candidiasis as they are also exposed to long term chronic treatment with antifungal agents.

Methods

Patient population: Isolates of *Candida spp* were collected from patients randomised into two prospective studies. Healthy women between the ages of 18-65 years with documented (culture proven) recurrent vaginal candidiasis (≥ 4 episodes per year), with no chronic underlying diseases (such as diabetes mellitus, immunodeficiency syndrome, etc), nor receiving any immunosuppressive medications or chronic antibiotics, were studied. These studies were approved by the hospital's Human Subjects Committee and written consent was obtained from each patient.

In study (A) all patients (women) were treated with ketoconazole 400 mg daily for 7 days, and half the male partners were treated, to assess the value of treating the sexual partners. In study (B), women were randomised to receive two forms of chronic suppressive therapy: (i) Itraconazole 200 mg orally daily for 5 days, then twice weekly for 6 months; or (ii) Clotrimazole 200 mg intravaginally daily for 5 days, then 200 mg twice weekly for 6 months. At the beginning of each study, all patients were suffering from acute symptomatic vulvovaginal candidiasis that was confirmed by both direct microscopy and culture. At the initial and subsequent visits, specimens were obtained by sterile cotton swabs from the vulva, vagina, rectum and oral cavity. Washings of the vagina, for quantitative cultures, were also obtained following irrigation with 5 ml of sterile saline. Repeat cultures from all four sites were obtained 1-2 weeks after the initiation of therapy, and monthly, thereafter, for a total of 12 months. Additional cultures were obtained if any patient developed a symptomatic recurrence of vaginitis. All specimens were inoculated onto Sabouraud glucose agar plates, and vaginal specimens were also inoculated onto chocolate agar. Typically-appearing colonies of candida growing on agar plates were Gram-stained to verify their identity as yeasts. Isolates were identified as *Candida albicans* by the presence of germ tube production in human serum and the production of chlamydospores in corn meal agar. Coded isolates of *C. albicans* were stored in 40% glycerol at -70°C and subcultures to

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Table Susceptibility of candida isolates (IC_{50} for 90%) before, during and after therapy

	Before (n = 110) IC_{50} mg/l	During (n = 50) IC_{50} mg/l	After (n = 94) IC_{50} mg/l	Range
Ketoconazole	0.04	0.04	0.04	0.02–0.63
Itraconazole	0.04	0.02	0.04	0.02–0.04
Clotrimazole	0.04	0.04	0.04	0.02–0.08

Sabouraud dextrose agar at 35°C for 48 hours prior to susceptibility testing. Isolates were tested blind and in duplicate.

Antifungal Agents: Pure powders of ketoconazole and itraconazole (Janssen Pharmaceuticals, Belgium), and clotrimazole (Miles Laboratories, Toronto, Canada), were dissolved in 100% dimethylsulfoxide to provide master dilutions of 1000 mg/l which were stored at –70°C.

Susceptibility Testing Procedure: A broth macro-dilution method⁷ was utilised with modification to improve endpoint reading.⁸ Inocula were prepared from a suspension of yeast colonies in sterile water containing 0.05% tween 80 which was vortexed and adjusted spectrophotometrically (90% transmittance at 530 nm) to correspond to 10^6 colony forming units/ml by viable count. Fifty microlitres (final concentration 2.2×10^4 cfu/ml) were added to twofold dilutions of the drugs ranging from 0.02–10.0 mg/l in yeast nitrogen broth. Some strains of yeasts were also tested in RPMI-1640 media for comparison. Drug/organisms suspensions were incubated at 35°C for 48 hours. Following vortexing to obtain an even suspension, percentage transmittance at 530 nm was determined spectrophotometrically. The inhibitory concentration was obtained by determining the lowest drug concentration at which 50% inhibition occurred compared with a drug free control. The 50% inhibitory endpoint concentration (IC_{50}) was defined as the lowest drug concentration satisfying the equation $\%T > T_c + 1/2(100 - T_c)$ where T is the transmittance of the test and T_c the transmittance of a drug-free control.⁸ Isolates obtained pre-, per- and post-therapy from the same patients were usually run on the same day. Resistance was defined as a greater than four-fold increase in baseline IC_{50} of post treatment isolates compared to pre-treatment isolates.

Results

Two hundred and fifty isolates of *C. albicans*, and four isolates of non-*C. albicans* strains were recovered and tested. Of the 110 candida isolates obtained before treatment, 90 were recovered from the vagina or vulva, 15 from the rectum and five from the mouth. Fifty candida isolates were obtained during treatment and of these, 40 were recovered from the vagina/vulva, seven from the rectum and three from the mouth. Of the 94 candida isolates obtained after treatment, 78 isolates were from the vagina/vulva, 10 from the rectum and two from the mouth. No significant difference in the results was observed between the two

media tested. The range of IC_{50} values was confined to a relatively narrow range at the low end of concentrations for all three drugs (table). There was no significant changes in the IC_{50} for any of the drugs tested for 90% or more of isolates obtained before, during or after therapy (table). Only one isolate obtained from the vulva showed a significant increase in the IC_{50} post-therapy over baseline, from 0.02 mg/l to 0.63 mg/l. However, this was considered an aberrant result as the isolates from the vagina and elsewhere did not show greater than four fold increase in IC_{50} .

Discussion

Recurrent vaginal candidiasis could possibly result from a clinically inapparent focus in the vagina. Vaginal relapse due to failure to eradicate the yeast with therapy despite clinical relief of symptoms has been postulated. Even though post therapy cultures are often negative, with clinical improvement, a single vaginal swab may be inadequate to detect low numbers of organisms,⁹ and may not confirm total elimination of yeast from the vagina. It is also unclear whether superficial invasion of yeast cells may result in the transition to an intracellular phase of the yeast with subsequent re-emergence weeks later as recolonization and infection of the vagina. Another popular theory to explain recurrences is reinfection from an intestinal reservoir.¹⁰ However, drugs such as ketoconazole and itraconazole should theoretically eradicate organisms from the intestinal reservoir and vaginal epithelial cells. Thus, testing for the development of in vitro resistance to explain persistent colonisation and reinfection could provide important information.

AIDS patients with recurrent oropharyngeal candidiasis have a somewhat similar problem, requiring long term suppressive therapy to prevent recurrences. Recent reports^{5,6} have documented the development of in vitro resistance associated with clinical failure to both ketoconazole and fluconazole.

Our study has shown that development of in vitro resistance to imidazoles in women treated with long term suppressive therapy is usually not seen. This is similar to the previous report of Sobel¹¹ where increased resistance to ketoconazole was not found in a similar group of patients, although only 40 isolates were tested. Similarly, Takada *et al*¹² noted no increased resistance to clotrimazole, although there was relative increase in recovery of *Torulopsis glabrata* with MIC values four times as high as those against *C. albicans*. While there is a general lack of correlation between in vitro sensitivity and invivo response with the imidazoles,¹³ recent reports in AIDS patients as previously discussed have found a correlation with in vitro resistance and clinical failure or recurrences.

The value of in vitro testing of fungi has been questioned also because of lack of standardisation of the method and inter-laboratory variation.¹⁴ However, although there is lack of reproducibility of results between laboratories

testing the same organisms, the reproducibility within the same laboratory is generally consistent.¹⁴ The method chosen in our study has overcome some of the concerns in antifungal susceptibility testing, such as trailing endpoints with subjective variability in the reading of fine haze,¹⁵ and also the results are independent of inoculum size.⁸ Moreover the relatively narrow range of inhibitory concentrations and lack of significant changes between isolates obtained before and after therapy speak for themselves. Although there may be difficulty and controversy in interpreting isolated fungal susceptibility results, our results with measurement of IC50 from isolates obtained from the same patient at different times and performed on the same day should be more reliable.

In summary there is no evidence that development of drug resistance plays a role in recurrent vulvovaginal candidiasis.

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